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Short communication

Shewanella oneidensis MR-1 Msh pilin proteins are involved in extracellular electron transfer in microbial fuel cells

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ABSTRACT

Shewanella is a microbial genus that can oxidize lactate for the reduction of insoluble electron acceptors. This reduction is possible by either direct (cell–surface interaction, nanowires) or indirect (soluble redox mediators) mechanisms. However, the actual molecular identification of a nanowire has not been determined. Through mutational studies, *Shewanella oneidensis* MR-1 was analyzed for its ability to transfer electrons to an electrode after deletion of the structural pilin genes (Δ*mshA-D*) or the entire biosynthetic expression system (Δ*mshH-Q*) of one of its pilin complexes (Msh type IV pilus gene locus). The complete removal of the Msh complex (Δ*mshH-Q*) significantly decreased the current generated from a fuel cell compared to MR-1. However, the mutant with only extracellular Msh structural proteins removed (Δ*mshA-D*) was able to generate 80% of the current compared to MR-1. Thus, the intracellular and membrane bound Msh biogenesis complex is a pathway for extracellular electron transfer in *S. oneidensis* MR-1.

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1. Introduction

Extracellular electron transfer (EET) by bacteria represents a phenomenon that occurs naturally during the biogeochemical cycling of metals in the environment [1–3] and was first exploited for generating electricity within mediator-less fuel cells in 2002 [4]. Bacteria from the Shewanellaceae and Geobacteraceae families represent the majority of strains that are currently used for understanding these mechanisms because of their current output and the body of work involving their cellular biochemical mechanisms [5,6]. These bacteria oxidize a wide array of short and long chain carboxylic acids to create reducing equivalents that can be externalized through outer membrane cytochrome cascades to both soluble and insoluble electron acceptors [5,7]. A better understanding of how charge is externalized from the outer membrane will elucidate possible mechanisms to aid in significantly increasing the current generated by microbial fuel cells (MFCs) and may also give an understanding of how these pathways can be transferred to other classes within the bacteria or archaea.

Currently, two pathways are presented for EET from dissimilatory metal reducing bacteria to an insoluble electron acceptor. These pathways can be defined by either the direct interaction with the electrode surface or indirect interaction through extracellular mediators [8,9]. These pathways are not mutually exclusive for *Shewanella oneidensis* MR-1, with both indirect (via biosynthesized mediators) and direct mechanisms occurring simultaneously [10–12].

In general, pili are non-flagellar polypeptide structures that can be expressed through a variety of secretion pathways. Pili were discovered on gram negative bacteria more than 50 years ago [13] and are implicated in cellular attachment, host–pathogen interaction, biofilm formation, cell–cell signaling, and genetic material transfer [14]. Recently, a new class of pilin-like appendages termed "bacterial nanowires" [15,16] were found to be conductive down the length of the pilin [17]. Considering the quantity and diversity of pilus-like appendages potentially expressed by *Shewanella* spp., the exact molecular classification of a "bacterial nanowire" remains unclear.

Several periplasmic and transmembrane proteins such as prepilin peptidases are required to express pili through pilin

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secretion pathways. These sub-membrane units are likely essential for the conduction of charge through a pilin-type appendage, but little to no work has been performed to elucidate their role. If bacterial nanowires play a significant role in electron conduction outside the membrane, either of the two major type IV pilin secretion systems in *Shewanella* spp. (msh and pil) should be responsible. Recently, MFC and biofilm data were reported for a diverse array of *S. oneidensis* MR-1 nanofilamentous-focused deletion mutants, indicating that Msh-type pili may play an important role in EET [18].

In this study, two different Msh mutants of *S. oneidensis* MR-1 were used: one lacking the complete biosynthetic system (Δ*mshH-Q*) and another lacking only the structural (extracellular component) proteins (Δ*mshA-D*). We compared the current generated by these two mutants in a miniature MFC (mini-MFC) to wild-type *S. oneidensis* MR-1. Our results indicate that the difference in the current output between the mutants compared to MR-1 can be attributed directly to the Msh pilin system. Therefore, this study identified the Msh pilin biosynthesis system as an integral pathway for extracellular electron transfer in *S. oneidensis* MR-1.

2. Materials and methods

2.1. Solutions, media, strains, and cell culture conditions

A stock solution of sodium lactate (1.95 M) was sterilized by autoclaving for 13 min at 121 °C and adjusted to pH 7. LB broth was used for liquid culture media (Difco). *S. oneidensis* MR-1 was purchased from ATCC (700550) and the two Msh deletion mutants were gifts from Dr. Daad Saffarini (University of Wisconsin-Milwaukee). In-frame chromosomal deletion of the Msh pilus biogenesis/pili complex (MR-1 Δ*mshHIJKLMNEGBACDOPQ* (Δ*mshH-Q*); Msh pili biogenesis mutant) genes has previously been described [18,19], and the same protocol was used to generate the in-frame deletion of the external Msh structural proteins (MR-1 Δ*msh*BACDO (Δ*mshA-D*); Msh structural pili mutant). *S. oneidensis* MR-1 and the two deletion mutants were grown from a −80 °C glycerol stock culture. After initial growth, each culture was transferred to 75 mL LB (1:100 dilution) in a 250-mL flask and incubated air-exposed at 25 °C with agitation at 100 rpm.

2.2. Bioinformatic study of Msh pilin complex

Two different bioinformatic analyses were performed to construct a hypothetical model of the Msh pilin complex from *S. oneidensis* MR-1. First, a bacterial protein localization prediction tool, PSORTb v.3.0 [20], was used to analyze the 16 MR-1 Msh pilin proteins (accession numbers in supplemental) encoded by the MR-1 Msh pilin gene locus. Secondly, sequence homology was obtained by comparison of the 16 MR-1 Msh pilin complex proteins to *Vibrio cholerae* O1 biovar El Tor (tax id: 686) using the BLAST-search algorithm [21] for proteins (BLASTp) with default algorithm parameters. Sequence homology of MR-1 Msh proteins to the well-studied Msh pilin complex from *V. cholera* [22] allowed for the same hypothetical predictions and locations of the proteins within both species.

2.3. Miniature MFC setup and data collection

The general dimensions (~2 cm^2 cross sectional area) and setup for the mini-MFC apparatus were described previously [23]. The electrodes within the fuel cell chambers were low-density graphite felt (0.13 g, Electrosynthesis Company, Lancaster, NY; 0.47 m^2/g) and were connected with titanium wires to an external load. Current density was reported using the electrode surface area. The anode and cathode chambers were separated by Nafion®-117 (The Fuel Cell Store). The anolyte and catholyte were passed through the chambers at a flow rate of 1–2 mL/min using a peristaltic pump. The catholyte for each fuel cell was a 50 mM potassium fericyanide solution in 100 mM phosphate buffer (pH 7.2) using uncoated graphite felt electrodes. All fuel cells were run at 25 ± 1 °C. Fuel cells used a 75-mL culture of *S. oneidensis* MR-1, Δ*mshH-Q*, or Δ*mshA-D* as the flow-through anolyte. Lactate was added to a concentration of 35 mM in each anode culture flask 24 h after the setup of the MFC. Voltages were measured across an external 680 Ω resistor and were recorded with a high-resolution data acquisition module (I/O tech, personal daq/54) every 2 min. All mini-MFC experiments with MR-1 and the mutants were performed in triplicate.

3. Results

3.1. Hypothetical model of Msh pilin complex

The lack of a Msh pilin complex model for *S. oneidensis* MR-1 has resulted in a lack of understanding of how this particular pilin complex is involved in EET. Therefore, a model was needed to understand the potential role of Msh genes. PSORTb v.3.0 [20] was used to analyze each of the 16 Msh pilin proteins to determine their predicted subcellular location. From this analysis, the predicted location of 6 Msh proteins (MshH, MshM, MshG, MshE, MshL, and MshB) was determined.

Secondly, a homology study of the predicted functions and locations of the well-studied Msh type IV pilin gene locus for *V. cholerae* [22] was performed. The homology of *S. oneidensis* MR-1 Msh pilin complex proteins were analyzed against *V. cholerae* O1 biovar El Tor (tax id: 686) via BLASTp [21]. Twelve of the 16 Msh proteins in MR-1 (MshH, MshI, MshJ, MshL, MshM, MshN, MshE, MshG, MshB, MshA, MshC, and MshD) had homology to an annotated Msh pilin protein from *V. cholerae*. Moreover, MshO, MshP, and MshQ from MR-1 had homology to three proteins in *V. cholerae*, which were annotated as hypothetical (NP_230066, NP_230067, and NP_230068, respectively). When a BLASTp search was performed on the hypothetical *V. cholerae* entries, the proteins were >98% identical to annotated Msh proteins in other *Shewanella* species. Lastly, MshK from MR-1 did not have homology to any Msh proteins in *V. cholerae.*

A schematic representation of the MR-1 Msh gene locus compared to *V. cholerae* is shown in Fig. 1. The order of the genes is identical within the two genomes, and the sizes are similar. When comparing protein identity of the homologs, all but one protein (MshK) is >25% identical, with five proteins (MshL, MshM, MshE, MshG, and MshA) having greater than 45% identity. Based on the homology between the Msh pilin complex from MR-1 and *V. cholera* and the PSORTb analysis, a working hypothetical Msh model for *S. oneidensis* MR-1 was created (Fig. 2). Eleven proteins were predicted to comprise the base of the Msh pilin complex. Eight proteins were predicted to be located in the cellular membranes, with three proteins (MshQ, MshL, and MshJ) located in the outer membrane and five proteins in the inner/cytoplasmic membrane (MshH, MshI, MshM, MshG and MshN). One protein was predicted to be located in the cytoplasm, MshE, based on PSORTb analysis. MshP and MshK were putatively assigned to the periplasm based on the hypothetical location of these proteins in *V. cholera*. Lastly, five Msh pili proteins were external structural proteins and therefore referred to as nanofilament proteins. These proteins included one major pilin protein (MshA) and four minor pilin proteins (MshB, MshC, MshD, and MshO).

3.2. S. oneidensis MR-1 and mutant culture characteristics

Growth curves of *S. oneidensis* MR-1 WT, Δ*mshH-Q*, and Δ*mshA-D* grown in LB were acquired through the use of Bioscreen C™ (Supporting Information, Fig. S1). Analysis of the growth curves showed similar trends between the wild type and the mutant cultures. The optical densities of the two mutants were within 10% of the wild type optical density throughout the growth curves with viable cell concentrations between 1×10^8 and 2×10^8 CFU/mL.

3.3. Current production by S. oneidensis MR-1 and Msh pilin mutants

The existence of conductive pilin (or nanowires) in Shewanellaceae and Geobacteraceae families has been reported previously [15,16]. A recent study reported MFC and biofilm data from several *S. oneidensis* MR-1 mutants. A modular voltage-based screening assay (VBSA) was used to show that when MR-1 mutants lack the

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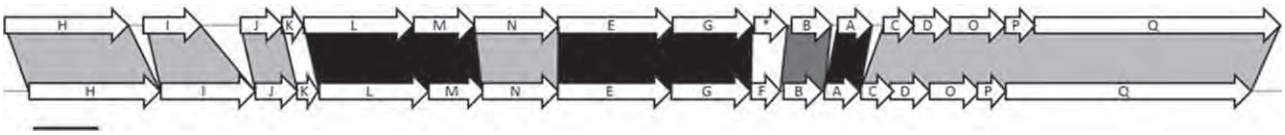

Fig. 1. Schematic representation of the predicted Msh gene locus in *S. oneidensis* MR-1 (top) and *V. cholerae* El Tor (bottom). Bold line below schematic represents 1000 nucleotides. Shading is based on identity at the protein level. Black shading, >45% identity; dark grey, 35–45% identity; pale grey, 25–35% identity; white, no homology.

genes *mshH-Q* (Δ*mshH-Q* and Δ*mshH-Q*/Δ*pilM-Q*), the DC current levels reached only 50% of wild-type output by the end of the experiment (150 h) [18]. Furthermore, compared to the amount of current generated from MR-1, the deletion of the flagellum (Δ*flg*) or the type IV pili intracellular biogenesis system (Δ*pilM-Q*) resulted in an increase in current output. The only knock-out mutants that decreased the current output, implying a potential role in EET for those knocked out genes, were the expected result from deleting the outer membrane cytochromes and the unexpected result that the Msh nanofilament and/or biogenesis proteins may also be involved [18]. However, since wild-type MR-1 can form a thick biofilm on the electrode, unlike the Δ*mshH-Q* mutant, the difference in current output in that system could have largely been due to biofilms and not the ability of the cells to transfer electrons to the outer membrane. Therefore, the mini-MFC system was utilized to negate the impact of differential biofilm formation on the electrode surface. A time-dependent wild-type MR-1 biofilm study was performed to validate this theory using the mini-MFC design at an external resistance of 680 Ω (Supporting Information, Fig. S2).

To better understand the role of the Msh pilin complex in EET, the mini-MFC design was used to compare current output from two different *S. oneidensis* Msh mutants to wild-type MR-1. The two mutants contained deletion of either the structural (nanofilament) or entire complex (nanofilament and base) of the Msh pilin system. Upon addition of 35 mM lactate in the mini-MFC system, MR-1 rapidly responded and was able to convert lactate into a DC output (Fig. 3 and Supporting Information, Fig. S3). Once lactate was added to WT MR-1, the current density immediately increased by 0.8 μA/cm^2 (0.4–1.2 μA/cm^2) and was sustained for ~20 h before starting to decline, eventually reaching its pre-lactate baseline at ~40 h. The Δ*mshA-D* mutant had a 0.3 μA/cm^2 increase in current density immediately after lactate addition, and continued to increase in current density over the next ~20 h until it reached a maximum of 1.0 μA/cm^2. The difference in baseline and maximum current density (~0.8 μA/cm^2) for the Δ*mshA-D* mini-MFC was exactly the same as for the wild-type control experiment. After the maximum current density was observed, there was a sharp decline in current production. Unlike wild-type MR-1and Δ*mshA-D*, the Δ*mshH-Q* mutant was only able to increase the current density by ~0.2 μA/cm^2 upon lactate addition, reaching a maximum value of ~0.4 μA/cm^2. The Δ*mshH-Q* mutant was able to sustain this substantially lower current density for ~30 h before decreasing to the pre-lactate baseline current density.

The mini-MFC data show similar maximum current output from both wild-type MR-1 and the Δ*mshA-D* mutant, indicating that the MshA-D proteins of the Msh pilin nanofilament are not needed to reach current output levels equal to that of the wild-type. However, it is possible that another nanofilamentous appendage (i.e. *pilA*) uses the Msh pilin base to deliver electrons outside the membrane. As such, the current output was reduced without the Msh pilin nanofilament immediately after lactate addition, but over time and with sufficient electron donor (lactate), the Δ*mshA-D* mutant was able to generate current at a similar level to wild-type MR-1. When the entire Msh complex was removed (biogenesis base and nanofilament proteins), as seen with the Δ*mshH-Q* mutant, the cells were not able to efficiently externalize electrons to the outer membrane.

Extracellular redox mediator concentrations (i.e. riboflavin) for wild-type MR-1 and Δ*mshH-Q* fuel cells have previously been reported [18] and were found to be similar in concentration within

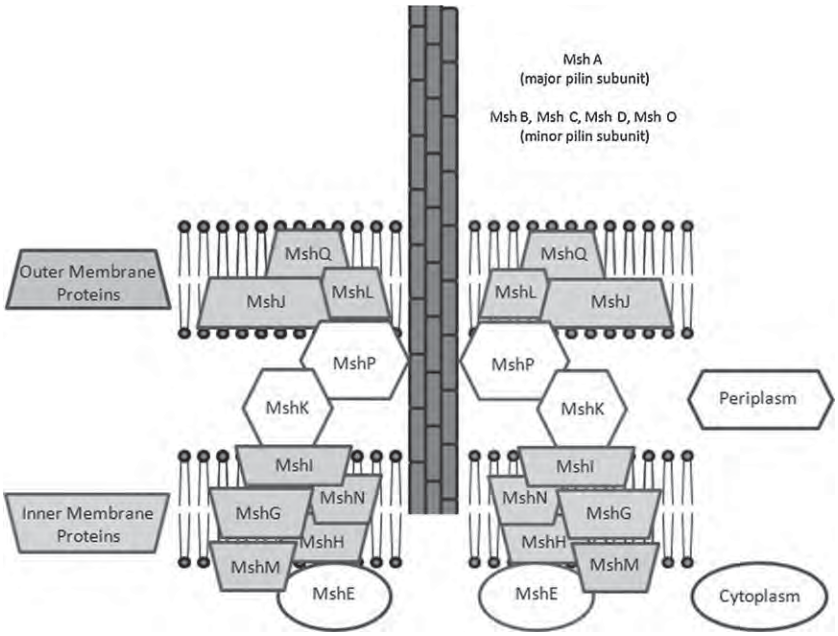

Fig. 2. Hypothetical Msh pilin model for wild-type MR-1.

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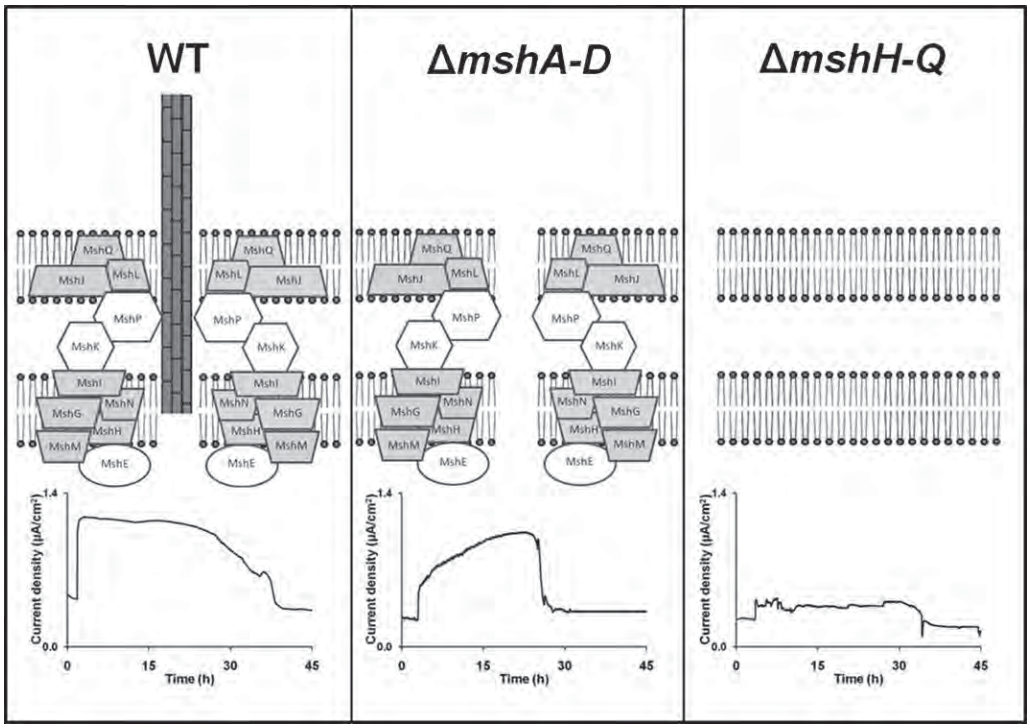

Fig. 3. Comparison of hypothetical *S. oneidensis* MR-1 Msh pilin complex in wild-type and Msh mutants to current output.

the Δ*mshA-D* culture supernatants (411 ± 20 nmol/L) in this work (data not shown) at the end of the MFC experiments. Therefore, the changes in mini-MFC current were not due to differences in extracellular redox mediator concentrations for the different Msh mutants. It is important to note that even with mediators, such as biosynthesized riboflavin, Δ*mshH-Q* was unable to generate substantial current in the mini-MFC. These data indicate that even with redox mediator molecules, *S. oneidensis* could not efficiently transfer electrons outside the membrane when the Msh biogenesis (base) complex was missing. There were also no significant differences between the rate of lactate utilization between the mutants and MR-1 as well (Supporting Information, Fig. S4).

Polarization curves were generated by changing the external resistance of the circuit for all mutants used in this study and compared to wild type MR-1. The open circuit potential for all cultures in the mini-MFC was between 0.75 and 0.81 V vs. ferricyanide/ferrocyanide (E° = 0.358 vs. NHE) (Fig. 4, inset). The short circuit current (I_{sc}) for MR-1 and Δ*mshA-D* was 1.1 mA and 0.99 mA, respectively. While the I_{sc} for the Δ*mshH-Q* mutant was 0.31 mA (Fig. 4). The amount of power generated by each culture from the mini-MFC decreased with MR-1 > Δ*mshA-D* > Δ*mshH-Q* (0.55 mW, 0.4 mW and 0.1 mW, respectively).

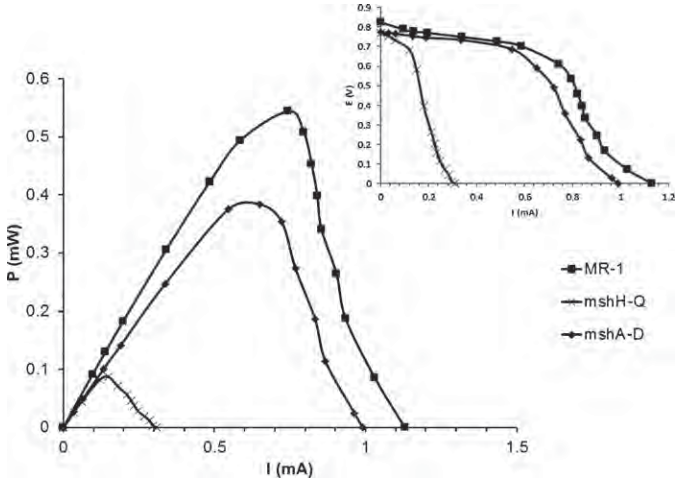

Fig. 4. Polarization curves for *S. oneidensis* MR-1 and two Msh pilin mutants, Δ*mshA-D* and Δ*mshH-Q*, from mini-MFCs (inset: plot of voltage vs. current).

In conclusion, this study used a hypothetical Msh pilin model and current generation from mini-MFCs to demonstrate that MR-1 Msh proteins are involved in extracellular electron transfer. Direct comparison of current output from wild-type MR-1 and two Msh pilin mutants showed that when the structural nanofilament Msh pilin proteins are deleted (Δ*mshA-D*), *S. oneidensis* retains its ability to generate current through extracellular electron transfer, either through direct contact with the electrode or shuttled through biosynthesized mediator molecules. However, when the entire Msh pilin protein complex was deleted (Δ*mshH-Q*), *S. oneidensis* was unable to generate significant amounts of current, indicating that the biogenesis Msh pilin protein complex is an integral component of the EET mechanism. Our results indicate that Msh biosynthetic pilin expression system proteins (base) are more important to electron transfer than the actual extracellular structural pilin proteins (nanofilaments).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2011.10.029.

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